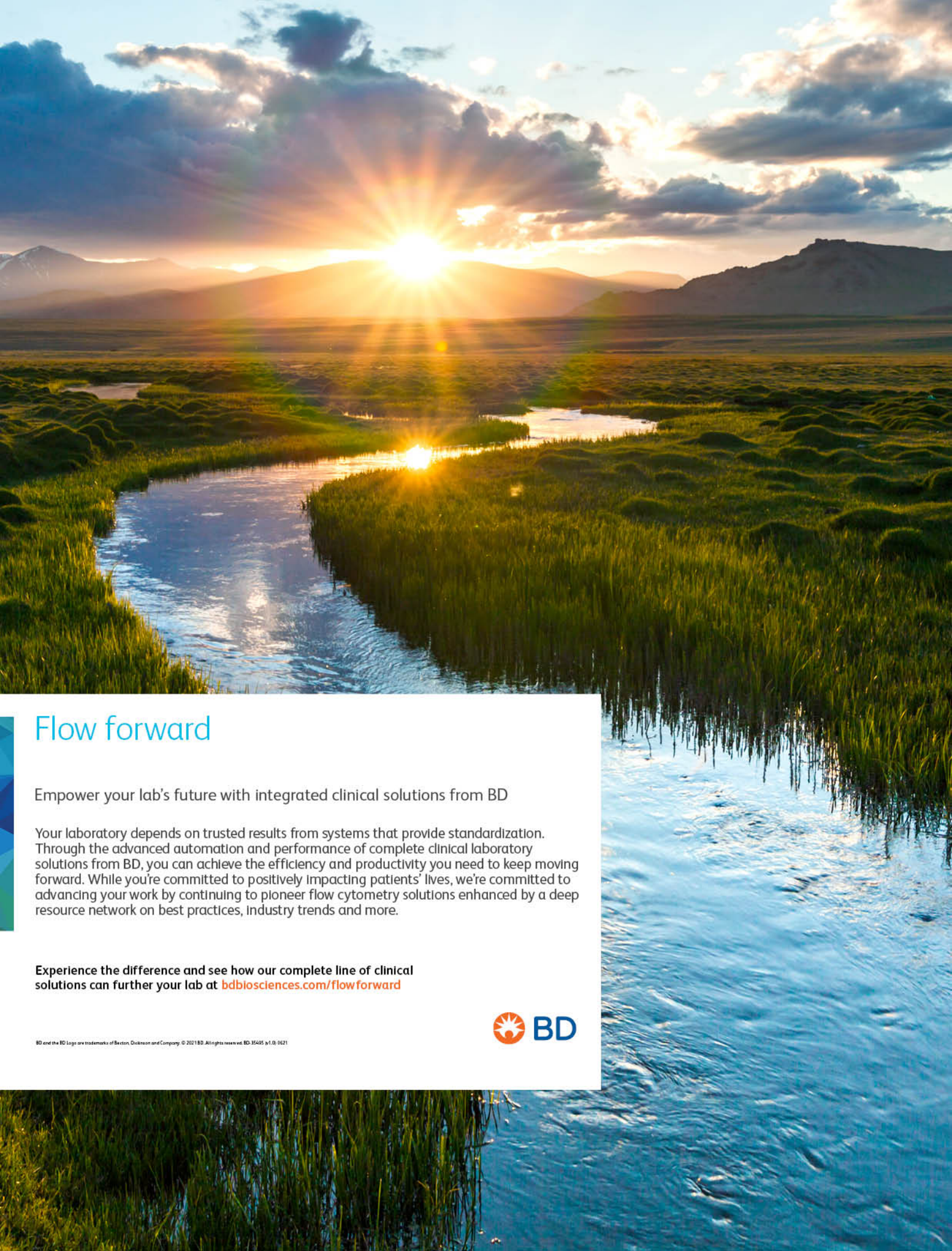

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REVIEW ARTICLE

CLINICAL CYTOMETRY WILEY

Flow cytometric assessment for minimal/measurable residual disease in B lymphoblastic leukemia/lymphoma in the era of immunotherapy

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Abstract

Minimal/measurable residual disease (MRD) is the most important independent prognostic factor for patients with B-lymphoblastic leukemia (B-LL). MRD post therapy has been incorporated into risk stratification and clinical management, resulting in substantially improved outcomes in pediatric and adult patients. Currently, MRD in B-ALL is most commonly assessed by multiparametric flow cytometry and molecular (polymerase chain reaction or high-throughput sequencing based) methods. The detection of MRD by flow cytometry in B-ALL often begins with B cell antigen-based gating strategies. Over the past several years, targeted immunotherapy directed against B cell markers has been introduced in patients with relapsed or refractory B-ALL and has demonstrated encouraging results. However, targeted therapies have significant impact on the immunophenotype of leukemic blasts, in particular, downregulation or loss of targeted antigens on blasts and normal B cell precursors, posing challenges for MRD detection using standard gating strategies. Novel flow cytometric approaches, using alternative strategies for population identification, sometimes including alternative gating reagents, have been developed and implemented to monitor MRD in the setting of post targeted therapy.

KEYWORDS
B-lymphoblastic leukemia/lymphoma, flow cytometry, minimal/measurable residual disease, targeted immunotherapy

1 | INTRODUCTION

Acute lymphoblastic leukemia (ALL) is a heterogeneous group of neoplasms with different clinical, morphologic, immunophenotypic, and genetic features and variable response to therapy (Swerdlow et al., 2017). ALL predominantly occurs in pediatric populations, with 75% of cases diagnosed before age of 6 years old. The 80%–85% of cases of newly diagnosed pediatric ALL have a precursor B-cell phenotype (B-ALL) and 12%–15% have a precursor T-cell phenotype (T-ALL) (Raetz & Teachey, 2016; Swerdlow et al., 2017).

Standard contemporary treatments include chemotherapy, radiotherapy, and hematopoietic stem cell transplantation (HSCT). The implementation of risk-stratified therapy has dramatically improved outcomes in pediatric ALL over the past several decades, with 5-year survival rates now reaching 80%–90% (Hunger et al., 2012; Pui et al., 2009). Minimal or measurable residual disease (MRD) is the most powerful prognostic factor in B-ALL and is predictive of relapse in both pediatric and adult patients, superseding other relevant prognostic factors including age, white blood cell count, and cytogenetics (Beldjord et al., 2014; Borowitz et al., 2008; Borowitz et al., 2015; Dhedin et al., 2015; Raff et al., 2007; Ribera et al., 2014; van Dongen et al., 2015). MRD has been applied in risk stratification and in informing risk-adapted therapies, including dose intensification or reduction of chemotherapy, HSCT, and novel therapies. While pediatric ALL has been considered a highly curable disease, disease relapse can occur after an initial response, and recurrent disease is often refractory to

conventional therapy and has a poor prognosis (Gokbuget et al., 2012; Gokbuget et al., 2016; Oskarsson et al., 2016). In adult patients, the cure rates are 40%–50% despite a high-complete remission rates of 80%–90% using the conventional regimens (Fielding et al., 2014; Sive et al., 2012). Therefore, efforts have been devoted to developing alternative therapeutic strategies to improve the efficacy against leukemic blasts.

2 | HISTORICAL PERSPECITVE: TARGETED THERAPY IN B-ALL

Progress in elucidating the interaction of immune system and cancer cells has led to breakthroughs in immunotherapy, which have substantial impact on patient outcomes. In relapsed or refractory (R/R) B-ALL, novel targeted immunotherapies have been introduced in both children and in adult patients and have shown encouraging results. The goal of targeted immunotherapy is to induce a tumor directed immune response upon binding to tumor antigens. There are currently four primary classes of targeted immunotherapy (Figure 1): (1) monoclonal antibody; (2) antibody-drug conjugate; (3) bispecific T-cell engagers (BiTEs); and (4) chimeric antigen receptor (CAR) T cell therapy (Zhou & Wang, 2021). The antigens targeted by immunotherapy in B-ALL are predominantly B cell markers expressed by leukemic blasts.

2.1 | Targeting B-cell markers in B-ALL

Surface CD20 expression is present in ~30% of B-ALL, defined as CD20 expression in ≥20% of blasts (Raponi et al., 2011). Anti-CD20 monoclonal antibodies (mAb) can bind to CD20 on leukemic blasts and induce leukemic cell death through various mechanisms, including complement dependent cytotoxicity (CDC), antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-dependent phagocytosis, and direct signaling induced cell death. After the anti-CD20 mAb rituximab was proven effective in B-cell non-Hodgkin lymphoma (NHL) and approved by the FDA in 1997, rituximab was incorporated into intensive chemotherapy regimens for CD20-positive B-ALL, with inclusion associated with improved outcome (Thomas et al., 2006; Thomas et al., 2010). The efficacy may be enhanced by upregulation of surface

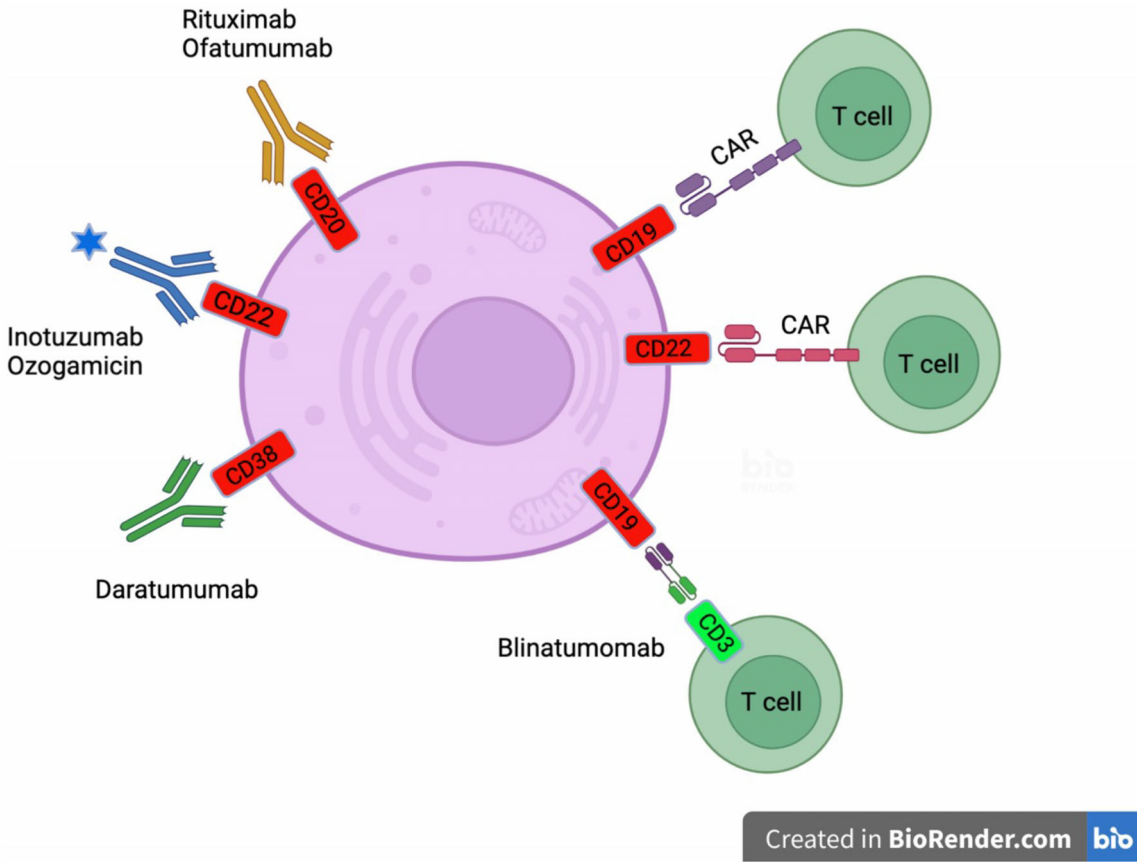

FIGURE 1 illustrates several targeted therapies that might be used in the treatment of B-lymphoblastic leukemia /lymphoma. These include monoclonal antibody based therapeutics including rituximab and inotuzumab ozogamicin which target CD20 and CD22, respectively; bispecific small molecules such as blinatumomab which engage CD3-positive T cells to target CD19-positive tumor cells; and CAR-T cells directed against B cell antigens. Patients with B- lymphoblastic leukemia /lymphoma may also be exposed to other targeted therapies such as daratumumab during the course of treatment which might not target tumor cells but will nonetheless alter the pattern of antigen expression of both neoplastic and normal background cells. [Color figure can be viewed at wileyonlinelibrary.com]

CD20 expression by chemotherapy and corticosteroids (Dworzak et al., 2010; Dworzak, Schumich, et al., 2008). Following rituximab, several additional anti-CD20 mAbs have been generated, two of which have been approved by the FDA. Ofatumumab binds to a different epitope than rituximab (Karlin & Coiffier, 2015), which may be beneficial in rituximab-resistant disease. The modification in Obinutuzumab creates better binding to immune effector cells (Pierpont et al., 2018). Additional randomized trials are needed to directly compare the efficacy among these anti-CD20 mAbs in different B cell neoplasms. There are several clinical trials in progress that incorporate use of anti-CD20 T cell engagers in mature B cell neoplasms(Liang et al., 2021), however these have not been implemented or FDA approved for use in B-ALL at the time of this writing.

CD19 is expressed throughout B cell maturation from hematogones to plasma cells and is expressed as well on the majority of B cell neoplasms including >90% of B-ALL (Raponi et al., 2011), making it a promising therapeutic target. Blinatumomab is a bispecific anti-CD19/CD3 T cell engager (BiTE) that consists of a small molecule with a physical bridge between fragments of antibodies directed against CD3-expressing T cells and CD19-expressing neoplastic cells to enable optimal interaction between these two cell types and thereby facilitate the anti-leukemia T cell mediated immune response. By bringing CD19-expressing leukemic blasts in close proximity with T cells, blinatumomab induces T cell engagement and ADCC to eliminate CD19-positive leukemic blasts (Franquiz & Short, 2020; Wang et al., 2021). In pediatric and adult patients with R/R B-LL, blinatumomab resulted in significantly improved survival rates compared with standard chemotherapy (Kantarjian et al., 2017; Locatelli et al., 2020; Locatelli et al., 2021). Currently, blinatumomab is the only therapy approved by the FDA for MRD-positive B-ALL following intensive chemotherapy. Ongoing studies are evaluating the role of blinatumomab in frontline therapy.

Immunotherapy using T cells genetically modified to express a CAR directed against CD19 has become one of the most promising therapeutic approaches in patients with R/R B-ALL. Anti-CD19 CAR-T cells have shown high efficacy in pediatric and adult R/R B-ALL patients with MRD-negative complete response achieved in 80–90% of patients (Curran et al., 2019; Frey et al., 2020; Gardner et al., 2017; Hay et al., 2019; Maude et al., 2014; Maude et al., 2018; Pan et al., 2017). The results of the ELIANA clinical trial led to the approval of tisagenlecleucel (Kymriah) for R/B B-ALL in children and young adults up to 25 years (Maude et al., 2018). In adult patients, the responses are not always durable in the absence of allogeneic HSCT (Hay et al., 2019; Park et al., 2018). There is significant variation among research groups with regards to CAR-T cell design and production, ultimately effecting the efficacy of a CAR-T cell construct. The role of tisagenlecleucel in the upfront setting is currently being explored.

Surface CD22 expression is present in >90% of B-ALL with uniform expression in ~80% of cases (Raponi et al., 2011). Inotuzumab ozogamicin (IO) is an antibody-drug conjugate, with anti-CD22 mAb conjugated to a cytotoxic agent calicheamicin. After binding to CD22 on the leukemic blasts, the CD22-IO complex is internalized and calicheamicin is released. Calicheamicin then induces double-strand DNA breaks and subsequent apoptosis of CD22-positive leukemic cells. In patients with R/R B-ALL, IO was associated with a higher rate of complete remission than standard of care (Kantarjian et al., 2016; Kantarjian et al., 2019). The role of IO in the upfront setting in B-ALL is under investigation. Other anti-CD22 mAb such as epratuzumab and the antibody-drug conjugate moxetumomab pasudotox have been studied in R/R B-ALL and showed modest clinical activity (Advani et al., 2014; Chevallier, Eugene, et al., 2015; Chevallier, Huguet, et al., 2015; Raetz et al., 2015; Short et al., 2018). CAR-T cells directed against CD22 have been developed and demonstrated clinical activity in B-ALL resistant to anti-CD19 immunotherapy with comparable potency to anti-CD19 CAR-T cells (Fry et al., 2018; Shah et al., 2020).

2.2 | Antigen escape after targeted immunotherapy

The common mechanism of acquired resistance to targeted immunotherapies is the modulation of expression of targeted antigens on leukemic blasts. Alteration of targeted antigen expression not only has an impact on the efficacy of the immunotherapy but also poses challenges to detection of MRD by flow cytometry due to phenotypic changes of leukemic blasts.

Decreased expression or loss of CD20 on the targeted population has been reported in ~25% of patients with persistent or relapsed B cell lymphoma following rituximab (Foran et al., 2001; Hiraga et al., 2009). Decreased or loss of CD20 after rituximab occurs by multiple mechanisms including genetic mutations in CD20, structural changes in binding region of the CD20 antibody, modulation of CD20 localization, and trogocytosis (Perez-Callejo et al., 2015). Downregulation or loss of CD19 on leukemic blasts has been reported in 8%–22% of patients with relapsed disease after blinatumomab (Jabbour et al., 2018; Mejstrikova et al., 2017). CD19-negative relapses have also been reported after anti-CD19 CAR-T cell therapy (Hay et al., 2019), and at higher rates after tisagenlecleucel treatment (Maude et al., 2018). Several mechanisms of CD19 antigen loss or escape have been proposed, including selection of preexisting alternative splicing at the CD19 locus resulting in truncated CD19 proteins lacking the specific epitope for binding (Safarzadeh Kozani et al., 2021; Sotillo et al., 2015), mutation of CD19 gene which alters the bindings properties and subcellular distribution of CD19 (Yu et al., 2017), selective expansion of CD19-/CD123+ leukemic blasts under immune pressure (Ruella et al., 2016), masking of CD19 epitope (Ruella et al., 2018), trogocytotic transfer of CD19 to CAR-T cells (Hamieh et al., 2019), or a lineage switch to acute myeloid leukemia (Gardner et al., 2016; Jacoby et al., 2016). As a result of trogocytosis, CD22 is downregulated after anti-CD22 mAb epratuzumab (Rossi et al., 2013).

To overcome immune escape and tumor resistance by antigen loss, bispecific CAR-T therapy targeting multiple antigens has been evaluated. A CAR product targeting both CD19 and CD22 has been

developed and demonstrated potent cytotoxic effects against leukemic blasts with promising clinical activities (Huang et al., 2020; Qin et al., 2018; Spiegel et al., 2021). A sequential CAR-T cell infusion strategy targeting CD19 followed by CD22 has established efficacy in pilot studies and showed high MRD-negative response rate and increased leukemia-free survival (Pan et al., 2020; Wang et al., 2020). Approaches to target additional leukemic antigens have also been attempted. Trivalent CAR-T cells targeting CD19, CD20, and CD22 provide enhanced cytolytic activity against CD19-negative blasts from patients who relapsed after anti-CD19 CAR-T cell therapy (Fousek et al., 2021). A dual CAR construct combining CD19- and CD123-mediated T cell activation has been developed and demonstrated superior in vivo activity against B-ALL comparing with single-expressing or pooled CAR-T cells, providing a potentially effective strategy to prevent and treat CD19-negative relapse after CD19-directed immunotherapies (Ruella et al., 2016). Advances in the development of novel targeted immunotherapies broaden the therapeutic spectrum of these therapies with improved efficacy and reduced toxicities, making targeted therapies a potential therapeutic modality to replace components of conventional chemotherapy.

3 | CURRENT STRATEGY FOR MRD DETECTION BY FLOW CYTOMETRY

The optimal MRD assay needs to be sensitive and accurate throughout the course of treatment, and allow implementation and standardization across laboratories. Multiparametric flow cytometry (MFC) and real-time quantitative polymerase chain reaction (RQ-PCR) for leukemia-specific rearrangements of immunoglobulin (IG) and T-cell receptor genes (TCR) genes are the most commonly used methods in clinical practice. High throughput next generation sequencing (HTS) has been gradually applied in MRD detection to improve sensitivity and specificity (Bashford-Rogers et al., 2016; Pulsipher et al., 2015). Compared to RQ-PCR and HTS based methods, flow cytometry is generally applicable to all B-ALL using standardized antibody panels, and offers rapid turnaround time for clinical decision making (Chen & Wood, 2017). Currently, MRD testing by flow cytometry is the standard of care in B-ALL in the United States.

3.1 | Immunophenotypic principles of MRD detection by flow cytometry

Flow cytometric identification of leukemic blasts and differentiation from normal lymphoid progenitors relies on the fundamental immunophenotypic principle that normal lymphoid progenitors show reproducible antigen expression patterns throughout maturation, whereas leukemic blasts show altered patterns of antigen expression secondary to underlying genetic mutations (Wood, 2016). Based on this principle, two related methodological approaches have been applied in MRD identification by flow cytometry.

The first approach is to identify a combination of antigens expressed on the leukemic blasts that are absent on normal lymphoid progenitors, referred to as "leukemia-associated immunophenotypes" (LAIPs) (Coustan-Smith et al., 1998). The commonly described LAIPs include antigen overexpression/underexpression, asynchronous antigen expression of progenitor markers and differentiation markers, homogeneous antigen expression, cross-lineage antigen expression, etc. (Dworzak et al., 2002; Vidriales et al., 2003). At diagnosis, LAIPs are identified using an extensive antibody panel and regions in multiparametric spaces are defined that contain only leukemic blasts but not normal progenitors. The informative antibody panel identified at diagnosis is then used in post therapy samples to recognize leukemic blasts with the specific LAIPs defined at diagnosis. This approach requires the knowledge of LAIPs at diagnosis to determine the informative antibody panel for follow up testing. It should be noted that therapeutic regimens may have an impact on the immunophenotype of both leukemic blasts and normal lymphoid progenitors, limiting the sensitivity and specificity of this method when used in isolation for MRD identification. Characteristic shifts in antigen expression with conventional and targeted therapies are detailed below.

An alternative approach, "difference from normal" (DFN), discriminates leukemic blasts from normal progenitors by recognizing immunophenotypic deviation of leukemic blasts from normal patterns of antigen expression (Cherian & Soma, 2021; DiGiuseppe & Wood, 2019; Wood, 2004; Wood, 2013). This approach can be used whether or not a diagnostic immunophenotype is available and relies on use of a standard antibody panel to differentiate leukemic blasts from normal regenerating B cell precursors. Using this approach, all progenitor populations at varying differentiation stages are evaluated for immunophenotypic aberrancies that deviate from normal antigenic patterns. Using the DFN approach, leukemic blasts can be recognized and distinguished from regenerating progenitors even in the setting of post therapies which may lead to immunophenotypic shifts on leukemic blasts. By contrast, use of a strict LAIP approach may lead one to overlook a leukemic population which has undergone immunophenotypic shifts due to therapy or at relapse. Although the DFN approach does not require knowledge of the diagnostic immunophenotype, if available, the LAIPs of the leukemic blasts seen at diagnosis may increase confidence that an identified putative abnormal blast population represents residual disease. DFN approach does require extensive expert knowledge of antigenic expression patterns on normal lymphoid progenitors in the resting and regenerative setting, making it more challenging than the LAIP method to standardize and implement across laboratories.

An integrated strategy simultaneously incorporating components of both approaches, is commonly applied to improve diagnostic accuracy in clinical practice. The assay sensitivity is dependent on the number of events collected, the number of events required to define a clonal leukemic blast population, the antibody panels used, the time points post therapy, and the degree of immunophenotypic deviation of the leukemic blasts from normal lymphoid progenitors. In a sample with abundant normal lymphoid progenitors, assay sensitivity may be significantly reduced, in particular if the diagnostic leukemic immunophenotype lacks specificity. In general, a sensitivity of 0.01% can be achieved in a large majority of B-ALL when 500,000–1 million events are collected (Borowitz et al., 2015).

3.2 | General approach to MRD detection by flow cytometry

The optimal MRD panel should include antigens expressed with varying intensity through maturation and antigens commonly aberrantly expressed on leukemic blasts. The optimal panel will also provide high-fluorescence intensities on cells of interest with low background and minimal antibody interaction. The selection of antigens for B-ALL MRD emphasizes the ability to recognize leukemic blasts with an aberrant, immature immunophenotype that deviates from normal lymphoid progenitors. A number of different antibody combinations have been tested in B-ALL MRD detection (Coustan-Smith et al., 2011; Dworzak, Gaipa, et al., 2008; Gaipa et al., 2012; Karawajew et al., 2015; Shaver et al., 2015). The Children's Oncology Group (COG) assay uses two 6-color reagent combinations for leukemic blast detection, and a third reagent combination provides a nucleated cell denominator for enumeration using a DNA/RNA binding dye (Syto16) (Borowitz et al., 2015). MRD measured by the COG assay as the percentage of nucleated mononuclear cells is associated with an adverse outcome in pediatric B-ALL and is useful in risk-stratification and risk-directed therapy [12]. The EuroFlow Consortium recently introduced a fully standardized 8-color two tube assay for B-ALL MRD testing (Theunissen et al., 2017). With adequate cells (>4 million) acquired, this assay allows separation between leukemic blasts and normal lymphoid progenitors in 99% of patients and reached a sensitivity of ≤0.001% (10^{-5}), comparable to RQ-PCR-based method. Recently, a high-sensitivity 10-color MRD assay has been described and demonstrated a sensitivity of 2×10^{-6} (0.0002%) with a median of 4,452,000 events collected (Tembhare et al., 2020), allowing for the detection of very low-level of MRD.

In the majority of described panels, CD19 is included as a principal B cell gating reagent to enrich for B lineage cells. CD19 is expressed through most stages of B cell maturation and is expressed on the majority of B cell neoplasms including B-ALL. The features that make CD19 an excellent B cell gating reagent make this antigen a common target by various immunotherapies. Both CAR-T cells and BiTE directed against CD19 deplete normal B cells and leukemic blasts expressing CD19 (Maude et al., 2015; Maude et al., 2018; Topp et al., 2015; Zugmaier et al., 2015). After anti-CD19 targeted immunotherapies, CD19 is insufficient to enrich for B cells; therefore, gating strategies need to be modified to include additional B cell markers to allow accurate B cell identification. A novel flow cytometric assay has been reported using both CD22 and CD24 as alternative B cell gating reagents and validated for MRD detection in the setting of anti-CD19 targeted immunotherapy (Cherian et al., 2018; Mikhailova et al., 2021). Approaches using additional B cell antigens such as cytoplasmic CD79a and approaches utilizing expression of CD10 or markers of immaturity have been described as well (Chen et al., 2022; Cherian & Stetler-Stevenson, 2018). Additionally, future studies could include evaluation of potential alternative pan-B cell markers such as CD40 and CD72 (Bishop & Hostager, 2001; Nitschke & Tsubata, 2004).

4 | IMPACT OF TARGETED IMMUNOTHERAPY ON DATA ASSESSMENT

4.1 | Impact of cytotoxic therapy

The immunophenotype of leukemic blasts is not always stable throughout therapy and at relapse. It has been reported that changes in expression of at least one antigen were observed in 69% of the cases between diagnosis and relapse (Borowitz et al., 2005) and 94% of cases between diagnosis and post induction MRD evaluation (Das et al., 2021). Antigenic expression shifts on leukemic blasts occur as early as day 15 during conventional induction therapy (Gaipa et al., 2005). The same phenotypic changes were observed in the normal lymphoid progenitors, suggestive of drug-induced phenotypic modulation (Chatterjee et al., 2021). A number of studies have shown that steroid treatment during induction therapy in patients with B-ALL can alter the immunophenotype of leukemic blasts, including decreased expression of immature antigens CD10, CD34, and CD38 and increased expression of mature antigens CD20, CD45, and CD73 (Arumugam et al., 2022; Dworzak et al., 2010; Dworzak, Schumich, et al., 2008; Gaipa et al., 2005; Gaipa et al., 2008). Such phenotypic changes on leukemic blasts may affect MRD recognition and lead to false negative results if rigid gating strategies with defined regions are solely used to identify LAIPs. During regeneration, the background normal B cells show left shifted maturation with proportionately increased hematogones, which may alter the normal lymphoid maturation pattern and has the potential to impact MRD assessment using the DFN approach. A combination of LAIP and DFN is therefore recommended for accurate MRD detection.

4.2 | Impact of targeted immunotherapy

Targeted immunotherapy leads to immunophenotypic changes on leukemic blasts and background normal lymphoid progenitors which differ from changes typically induced by conventional chemotherapy and as noted previously, often result in a reduction of expression of the targeted antigen on both normal and abnormal populations. The changes seen may be dependent on the mechanism of action of the therapeutic agent, with monoclonal antibodies showing different patterns and kinetics of antigenic shift than is seen with T cell engaging therapies. Recognition of characteristic shifts in antigen expression is critical for accurate interpretation of flow cytometric data in the post therapy setting.

As CD20 targeting monoclonal antibodies have been in clinical use for over 30 years, there is significant data regarding the impact of anti-CD20 mAbs on antigen expression. In patients with non-Hodgkin lymphomas treated with anti-CD20 mAb rituximab, B cell maturation arrest occurs at the early stage 2 hematogone and is accompanied by complete depletion of naïve, mature B cells (Carulli et al., 2015) (Figure 2). These changes were reported as short-term effects, with complete normalization of B cell ontogeny 12 months after the last rituximab infusion (Carulli et al., 2015). Anti-CD20 therapy may also

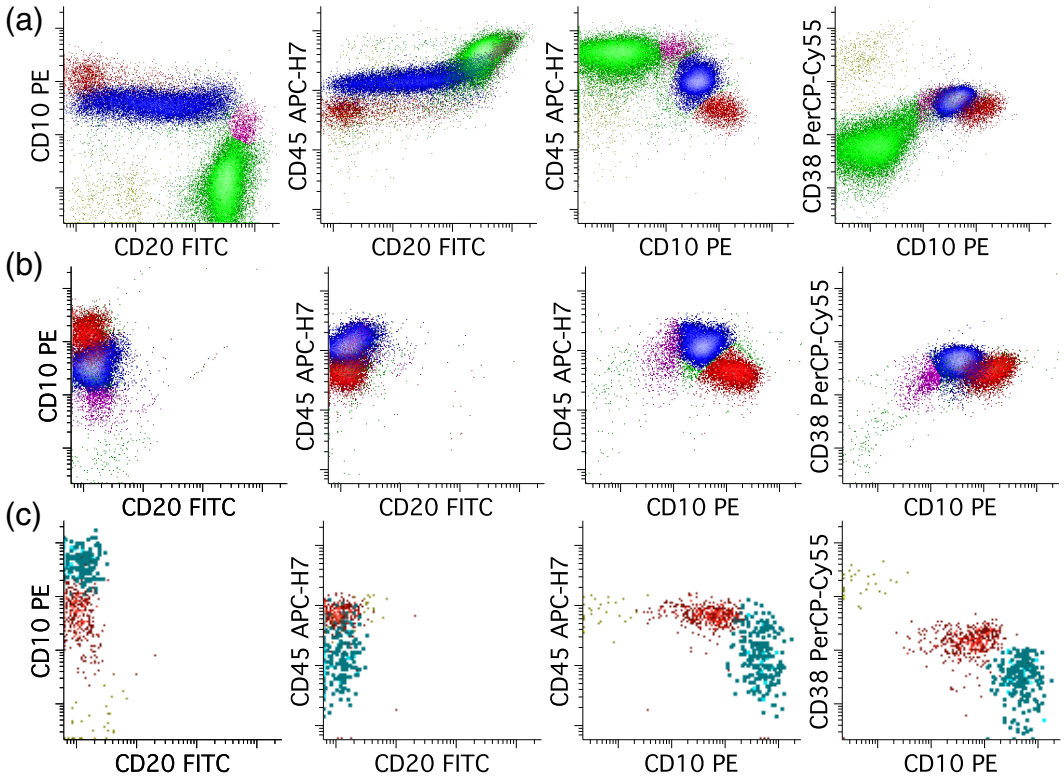

FIGURE 2 Impact of anti-CD20 mAb on normal B cell maturation and on leukemic blasts. All plots show all CD19-positive B cells from the bone marrow. (a) Normal B cell maturation. Stage I hematogones (colored in red) express bright CD10 without CD20. Gradual loss of CD10 and acquisition of CD20 occur in stage II hematogones (colored in blue). Expression of CD20 is higher in stage III hematogones (colored in pink) than in mature B cells (colored in dark green). (b) After rituximab therapy, CD20 is absent on stage I-III hematogones. When present, mature B cells will also lack CD20. (c) In this example from a patient with a history of B-lymphoblastic leukemia post anti-CD20 therapy, two dominant populations lacking CD20 are present. A population of stage one hematogones (colored in red) is present and in addition, a population of abnormal blasts is noted (highlighted in aqua) which aberrantly expresses bright CD10, dim to absent CD38, and dim to absent CD45. Both populations can be recognized and distinguished when using an adequate panel. [Color figure can be viewed at wileyonlinelibrary.com]

lead to reduction of CD19 expression (Jones et al., 2012), and in practice, reduced intensity of CD19 may be seen on B cells persisting post anti-CD20 therapy, a variable which can complicate use of CD19 as a B cell gating reagent. CD20 is expressed on ~30% of B ALL and when present may be expressed uniformly or in a heterogeneous fashion (Raponi et al., 2011). CD20 targeted mAbs may be incorporated into therapeutic regimens for B-ALL when CD20 expression exceeds 20% of leukemic blasts (Levato & Molica, 2018). Marrow specimens collected post anti-CD20 therapy may show a left shift in maturation with absence of CD20 expression on stage II-III hematogones as well as on mature B cells (Figure 2b). Residual B cell populations may appear arrested at an early stage of maturation, raising concern for residual disease in some projections. However, if adequate parameters are evaluated, true leukemic blasts can generally be differentiated from regenerating, CD20-negative B cell precursors present post anti-CD20 therapy (Figure 2c).

CD19 is the target of most FDA approved and commercially available T cell engaging therapies including bi-specific small molecules such as blinatumomab and cellular products such as CAR-T cells. CD19 directed T cell engagers typically lead to ablation of normal and abnormal B cells which continues while the T cell engager is in in the system. Blinatumomab has unique pharmacokinetic features including rapid clearance and a relatively short elimination half-life and must be administered as a continuous infusion (Franquiz & Short, 2020; Zhu et al., 2016), whereas the persistence and efficacy of CAR-T cells, with associated B cell depletion, are dependent on the molecular characteristics of the CAR constructs, prior chemotherapy, and lymphodepleting regimens (Maude et al., 2015; Pietrobon et al., 2021). Post CD19 CAR-T cells, unexpected patterns of recovery may be seen. For instance, a predominance of CD19 negative progenitors may be seen at early intervals after therapy (discussed further below) (Cherian et al., 2018) and some groups have described an increase in circulating hematogones (Xiao et al., 2018). Leukemic blasts in relapsed B-ALL post CD19 directed T cell engaging therapies may be CD19 negative or positive (Pillai et al., 2019). Figure 3 illustrates an example of a CD19 negative relapse. The impact of anti-CD19 therapy on background B cell progenitors and marrow recovery likely depends on both the mechanism of action and distance from therapy, with mAbs leading to different patterns of recovery compared to T cell engagers (Figure 4).

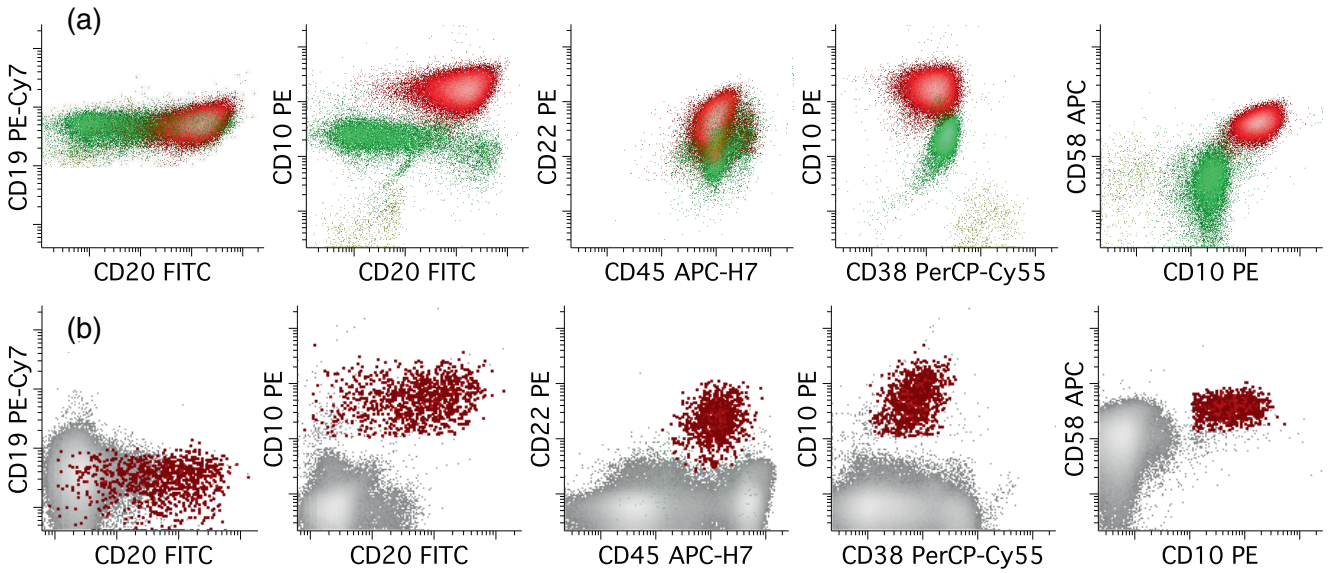

FIGURE 3 CD19 negative relapse post CD19-directed CAR-T cells. (a) Row 1: All plots show CD19-positive cells. Plots illustrate leukemic blasts (red) present in a patient with relapsed/refractory B-lymphoblastic leukemia, present in a background of left shifted hematogones (green). The abnormal blasts comprise ~7% of the white blood cells and demonstrate aberrant expression of CD10 (bright), CD20, CD38 (mildly decreased), and CD58 (increased). (b) Row 2: All plots show mononuclear cells. Although no CD19-positive cells are evident, an abnormal blast population is evident (0.25% of the white blood cells) that lacks CD19 but expresses B cell antigens CD20 and CD22 and shares the immunophenotypic abnormalities seen in this patient prior to therapy. The findings represent a CD19 negative relapse. [Color figure can be viewed at wileyonlinelibrary.com]

CD22 is expressed by >90% of B-ALL and may be expressed uniformly or in a heterogeneous fashion (Raponi et al., 2011; Shah et al., 2015). Reduced to absent expression of CD22 is described in some subsets of B-ALL such as B-ALL with a KMT2A rearrangement. *BCR/ABL* fusion may also influence CD22 expression, with decreased expression in B-ALL with a more mature phenotype. The impact of CD22 directed therapy on CD22 expression by the leukemic blasts is controversial with some studies suggesting a decrease in positivity and intensity of antigen expression with therapy (Kantarjian et al., 2021; Lanza et al., 2020) while other studies demonstrate that CD22 expression may be stably maintained over a prolonged period following therapy (Shah et al., 2015).

Loss of a single targeted antigen is one of several challenges to flow cytometric detection of MRD emerging in the setting of targeted therapies. It is important to note that targeted therapies may be used sequentially, and exposure to multiple targeted therapies may result in sequential loss of B cell markers on a leukemic blast population (Reinert et al., 2021). The selective pressure applied to a leukemic blast population by a targeted therapy may lead not only to antigen loss but to more profound changes in the characteristics of the leukemic blasts such as changes in lineage. Further, patients might be exposed to agents that are not specifically directed against the leukemic population but yet have the effect of altering the immunophenotype of the leukemic blasts and normal progenitors in the background, complicating MRD detection by flow cytometry (Chatterjee et al., 2021). Strategies for contending with such challenges and specific examples are included below.

5 | STRATEGIES FOR MRD DETECTION POST TARGETED IMMUNOTHERAPY

Post CD19-directed immunotherapy, CD19 is no longer a reliable gating reagent due to depletion of CD19-positive normal B cells and leukemic blasts and possible relapse with a CD19-negative immunophenotype. Novel gating reagents and strategies emphasizing redundancy and flexibility are required for the detection of residual leukemic blasts. Both CD22 and CD24 are differentially expressed in B lineage cells during maturation in bone marrow (Duperray et al., 1990; Hunte et al., 1998; Uckun et al., 1992), making them suitable alternative gating reagents to enrich for B cells. A novel flow cytometric assay using expression of either CD22 or CD24 as gating reagents to identify B lineage cells has been validated in MRD detection following anti-CD19 immunotherapy (Cherian et al., 2018). Using this panel, a "rough B cell gate" is defined to include either CD22- or CD24-positive cells without CD66b (Figure 5) and a standard DFN combined with LAIP approach is utilized to identify abnormal immature B cell populations. This approach successfully identified both CD19-positive and CD19-negative leukemic blasts and showed a good correlation with the standard flow cytometric B-ALL MRD assay.

It is critical to note that when altering gating strategies, it is important to re-establish normal patterns as differential gating strategies may include or exclude different populations. Compared to CD19 gating, some populations may be excluded using a CD22 or CD24 gating strategy. For instance, plasma cells, which normally express

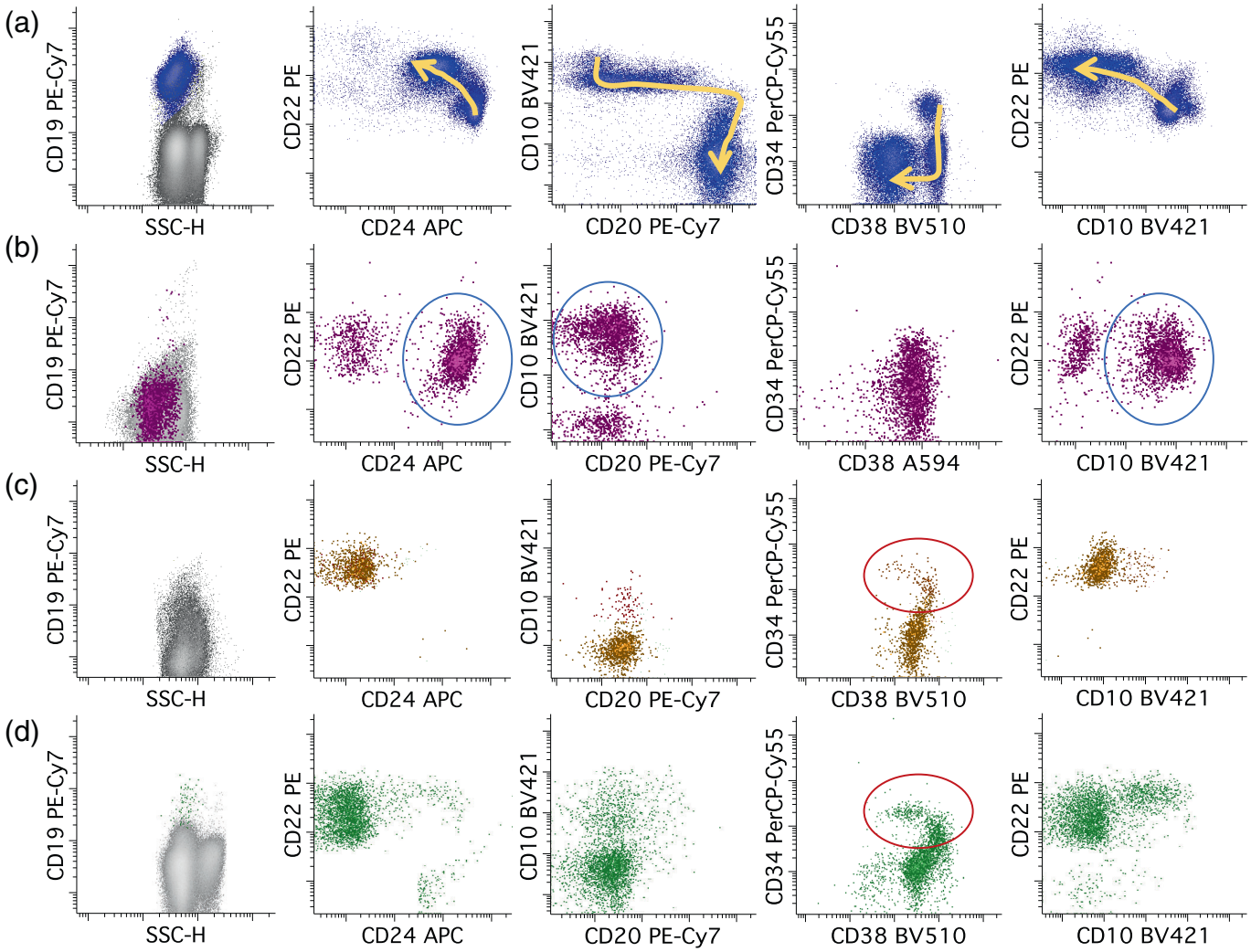

FIGURE 4 All plots show data from bone marrow flow cytometry studies from four patients at different intervals during therapy. All plots in column 1 show all mononuclear cells with CD19-positive B cells highlighted. Remaining dot plots show all cells positive for CD22 or CD24 without CD66b. Each row represents a different pattern of B cell recovery after therapy. (a) Post transplant. All stages of B cell maturation are represented. Arrows follow changes in antigen expression with maturation. (b) This sample was collected approximately 1 week after completion of therapy with Denintuzumab mafodotin (SGN-CD19A), an antibody drug conjugate targeting CD19. CD19 positive cells are essentially absent. Note the presence of B cell precursors that express CD22, CD24, and CD10 without CD19 or CD20 highlighted by the blue ovals. (c) This sample is collected 1 month post CAR-T cell therapy. Note the near absence of B cells expressing CD19. A small heterogeneous population is noted expressing CD22 without CD24. A subset of these cells (highlighted by the red oval) express variable, dim CD10 and CD34 and represent normal CD19-negative progenitors (discussed further in the text and in Figure 6). The remaining cells in this gate express CD22 but lack CD10, CD19, CD20, CD24, and CD34. These cells, represent a heterogeneous population that is not a progenitor and, though CD22 positive, is not of B cell origin. As noted in the text, CD22 expression is not specific to B cells. (d) The 3 months post CAR-T cell therapy. As compared to the time point illustrated in panel C, more B cell recovery is noted. Very few CD19-positive B cells are present but more cells are noted expressing CD22 with variable CD10, CD24, and CD34 (highlighted by the red oval) representing very early B cell progenitors. The remaining cells in this gate express CD22 but lack CD10, CD20, CD24, and CD34 and are of uncertain lineage but are not interpreted to represent either progenitors or B cells. [Color figure can be viewed at wileyonlinelibrary.com]

variable CD19, are negative for both CD22 and CD24 (Gilad et al., 2019; Horst et al., 2002) and will be excluded when using this approach. There are also notable populations which express CD22 or CD24 but lack CD19. Such CD19-negative populations will be identified in a CD22 or CD24 defined B cell gate and may include mature populations and progenitors. Recognition of such populations is critical to avoid misidentification of a normal population as an abnormal blast population. Two progenitor populations expressing CD22 without CD19 or CD24 have been described (Figure 6) and may be proportionally increased post anti-CD19 T cell engaging therapies. One expresses CD22 (dim), CD34 (bright), and CD38 (intermediate) without CD10, CD20, or CD24. This population may represent lymphoid-primed multipotent progenitors but additional evidence is needed for definitive lineage assignment for this population (Bueno et al., 2022).

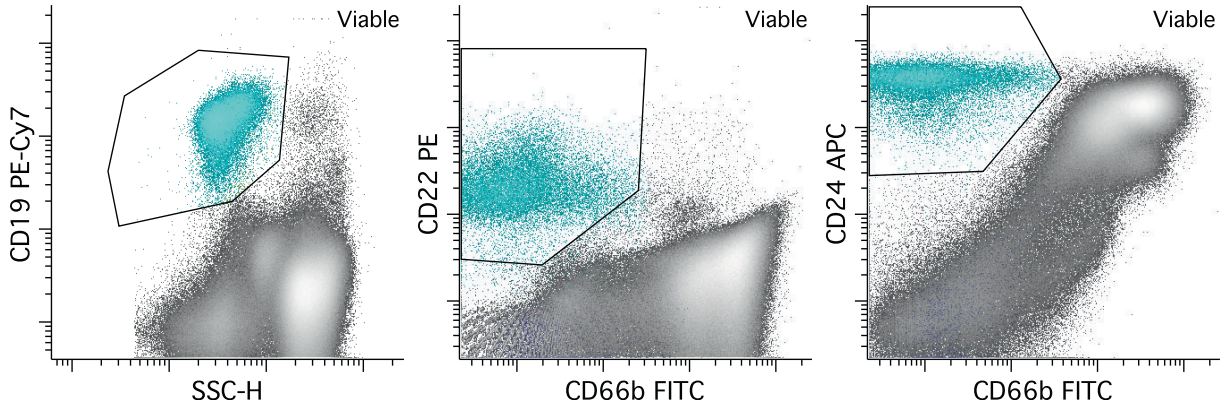

FIGURE 5 Alternative gating strategy to identify B cells using CD22 and CD24. In the setting of post anti-CD19 therapy, a combination of CD22 and CD24 can be used to gate B cells. [Color figure can be viewed at wileyonlinelibrary.com]

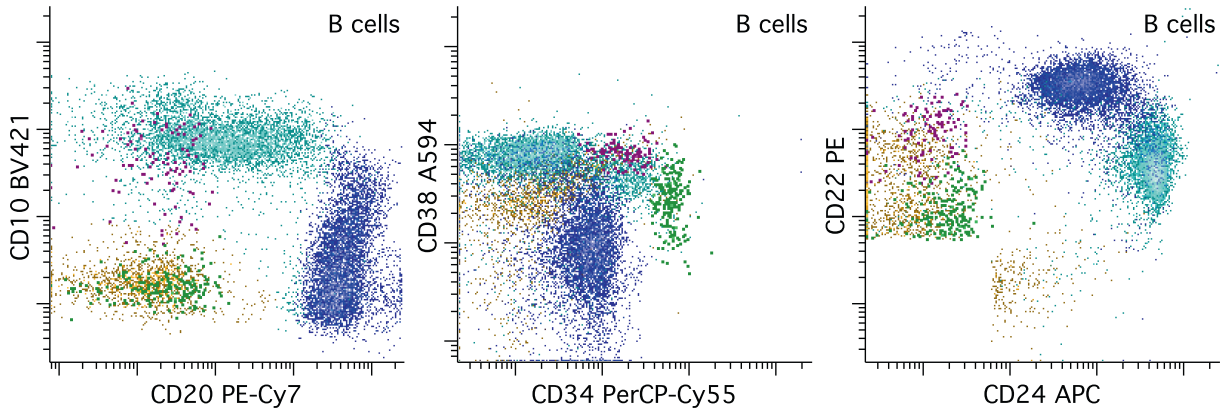

FIGURE 6 CD19-negative progenitor populations present in "B cell gate" using CD22 and CD24 as gating reagents. All plots show cells either CD22- or CD24-positive without CD66b. In addition to CD19-positive hematogones (aqua) and mature B cells (blue), two CD19-negative progenitor populations are identified in "B cell gate". The first population (highlighted in green) expresses CD22 (dim), CD34 (bright), and CD38 (intermediate) without CD10, CD20, or CD24. The second population (highlighted in pink) expresses CD10 (variable), CD20 (dim), CD22, CD34, and CD38 (bright) without CD24. [Color figure can be viewed at wileyonlinelibrary.com]

The other expresses CD10 (variable), CD20 (dim), CD22, CD34, and CD38 (bright) without CD24 and has been referred to by some as early lymphoid progenitors (Bueno et al., 2022; Mikhailova et al., 2021). Both populations are significantly increased in proportion post anti-CD19 T cell engaging therapies and should be recognized so they are not confused with neoplastic blasts. Additionally, normal mature populations, such as basophils (Toba et al., 2002), mast cells (Escribano et al., 1998), and plasmacytoid dendritic cells (Reineks et al., 2009) with variable expression of CD22, may be included in this gate. Careful attention to the clone of CD22 utilized is critical as some clones are more likely to bind populations including basophils and plasmacytoid dendritic cells (Reineks et al., 2009). Inclusion of additional markers of immaturity and antigens which may be aberrantly expressed on neoplastic B lymphoid blasts may help distinguish normal CD22-positive populations from leukemic blasts. Knowledge of the diagnostic immunophenotype can also be helpful in this regard. Because CD24 expression is aberrantly decreased or absent in a subset (~20%) of B-ALL (Cherian et al., 2018; Uckun & Song, 1993), in particular, B-ALL with a KMT2A rearrangement (Coustan-Smith et al., 2011; Schwartz et al., 2003), lack of CD24 may be an aberrant immunophenotypic feature of leukemic blasts. However, given the presence of normal CD24-negative/CD22 positive progenitors, caution needs to be taken when considering a CD22-positive population lacking CD24 identified using this gating strategy, in particular in the setting of recent anti-CD19 T cell engaging therapy. Use of a comprehensive panel, consideration of the diagnostic immunophenotype demonstrated by the leukemic blasts, and a thorough understanding of the immunophenotype of normal progenitor and mature populations is critical to avoid mis-interpretation of such populations as residual leukemic blasts. Anytime a gating strategy is altered, a thorough validation process, including evaluation of samples post common immunotherapies is critical to re-establish normal patterns and to understand the impact of therapy on normal maturation.

Additional strategies for identification of abnormal blasts include utilization of cytoplasmic CD79a for B cell identification (Chen et al., 2022; Cherian & Stetler-Stevenson, 2018). A recent study

showed that cCD79a gating could effectively detect MRD in B-ALL patients post CD19 CAR T cell therapy and predict outcomes after HSCT (Chen et al., 2022), providing preliminary evidence that cCD79 gating is a feasible option post targeted therapy. Alternatively, a more open flexible gating strategy using several antigens, often over expressed in B-ALL and evaluated against side scatter and/or CD45 can be attempted to evaluate for an abnormal population. It should be noted that such approaches may suffer from reduced sensitivity and specificity and there may be scenarios where molecular genetic strategies are required to achieve the desired assay performance.

In patients receiving multiple immunotherapies targeting different antigens, current gating reagents including CD19, CD22, and CD24 may be ineffective in enriching for B cells. For example, in a patient post concurrent anti-CD19 and anti-CD22 targeted therapies, both CD19 (Figure 7a) and CD22/CD24 (Figure 7b) based gating identified only a small number of B cells, including an abnormal immature population expressing CD10, CD22 (dim), and CD38 without CD24. However, gating using CD10 on all viable cells recognized a much larger abnormal population (Figure 7c), with a similar immunophenotype to that seen in the diagnostic sample. In patients post targeted therapies, in particular, with complex treatment history, samples need to be evaluated in a systematic way, with incorporation of multiple parameters (including B cell antigens and antigens associated with immaturity) to improve diagnostic accuracy.

6 | UNEXPECTED FINDINGS IN THE SETTING OF TARGETED IMMUNOTHERAPY

6.1 | Relapsed acute leukemia with lineage switch

Relapsed acute leukemia with lineage switch has been reported in a small percentage (0.6%) of pediatric patients, the majority of whom harbored a KMT2A rearrangement and switched from B lineage at diagnosis to myelo-monocytic lineage at relapse (Rossi et al., 2012). In B-ALL patients receiving CD19 directed T cell engaging therapies including anti-CD19 CAR-T cell therapy or blinatumomab, CD19-negative relapse with myelo-monocytic lineage switch has been identified at a relatively higher frequency. Most of these reported cases carried KMT2A rearrangement (Gardner et al., 2016; Rayes

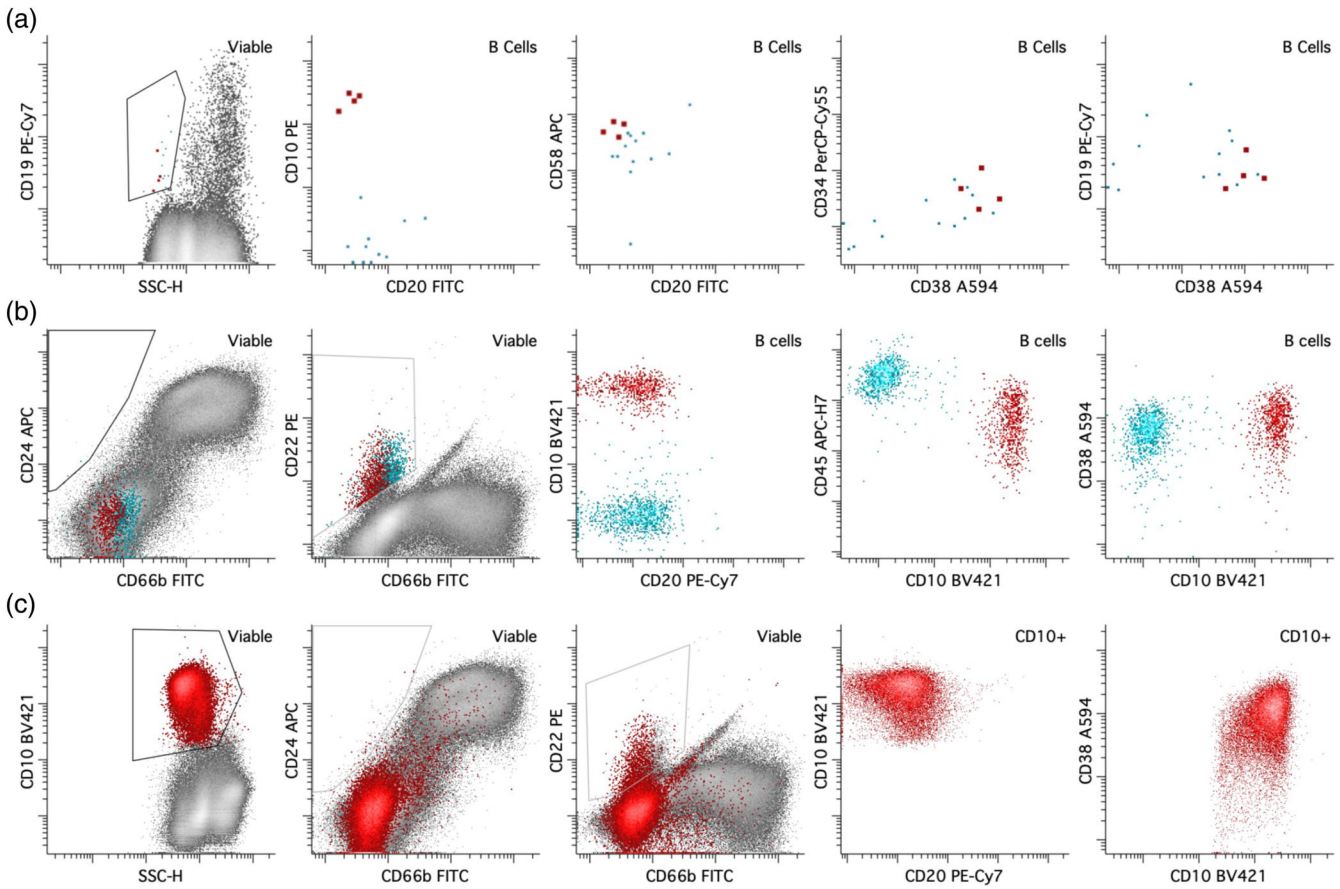

FIGURE 7 Alternative gating strategy to identify B cells after immunotherapies targeting different B cell markers. (a) Only few B cells are identified using CD19 gating. Red dots represent immature B cells with an aberrant immunophenotype suspicious for MRD. (b) There are more B cells identified by CD22 and CD24 gating than by CD19 gating. The subset colored red represents abnormal immature B cells with an immunophenotype similar to that seen in A. (c) Gating with CD10 generates a much larger population of abnormal immature B cells (colored red) with an immunophenotype similar to that seen in A and B. [Color figure can be viewed at wileyonlinelibrary.com]

et al., 2016), BCR-ABL1 (Nagel et al., 2017), or ZNF384 rearrangement (Oberley et al., 2018), and relapsed with acute myeloid leukemia harboring the same gene rearrangement as present in B lymphoblasts at diagnosis. The myeloid blasts showed either identical clonal immunoglobulin (IG) gene rearrangement as detected in B lymphoblast at diagnosis or germline configuration of IG genes.

Relapse with lineage switch represents a novel mechanism of resistance to anti-CD19 immunotherapy. The potential underlying mechanisms of lineage switch have been proposed, including selection of a pre-existing, unrecognized CD19-negative myeloid clone or uncommitted stem cells, and selective pressure leading to reprogramming/de-differentiation of a previously committed B lymphoid blasts to a myelo-monocytic blast (Gardner et al., 2016; Nagel et al., 2017). In a murine ALL model, downregulation of B cell transcription factors PAX5 and EBF1 during anti-CD19 immunotherapy drives the reprogramming of B lymphoid blasts and subsequent a CD19 negative relapse, supporting lineage switch as a mechanism of therapeutic resistance (Jacoby et al., 2016). Standard B-ALL MRD assays can detect residual abnormal lymphoid blasts at a sensitivity of ~0.01%. However, these panels are not adequate to detect aberrant myeloid or monocytic blast populations, unless the aberrant blasts are significantly expanded, and additional assays for myeloid and monocytic cells are required in cases concerning for lineage switch. Molecular methods such as HTS for IG gene rearrangements would be useful to tract the dominant sequences identified at diagnosis and monitor the newly emerging clones. It may be prudent to follow patients with a genetic profile more susceptible to lineage switch post anti-CD19 T cell engaging therapy with myelo-monocytic antigen evaluation and with genetic markers of residual disease. An example of lineage switch post blinatumomab is illustrated in Figure 8.

6.2 | Targeted therapies received for conditions other than B-ALL

Targeted therapies have a number of established applications and may be used in patients with acute leukemia for applications beyond targeting the tumor. Even in the neoplasm that is not the direct target of a directed agent, such targeted therapies can impact the immunophenotype of the neoplastic or of background cells. Information about targeted therapies not directed against the tumor may not be listed on a requisition; however, recognition of such phenomenon is critical so as to not mistake the impact of targeted therapies used in these settings for residual disease.

Daratumumab, a human IgG kappa mAb against CD38, has been widely used in the treatment of multiple myeloma (Costello, 2017). Newer anti-CD38, such as isatuximab, can induce direct apoptosis of neoplastic cells (Deckert et al., 2014). Recently, it has been reported that daratumumab is a potentially effective therapy in autoimmune hemolytic anemia (AIHA) in pediatric patients post HSCT (Schuetz et al., 2018). Daratumumab may saturate the CD38 molecules on all CD38-expressing cells throughout the body for several months following discontinuation of treatment (Oberle et al., 2017). Because the standard diagnostic CD38 antibody binds to epitopes overlapping

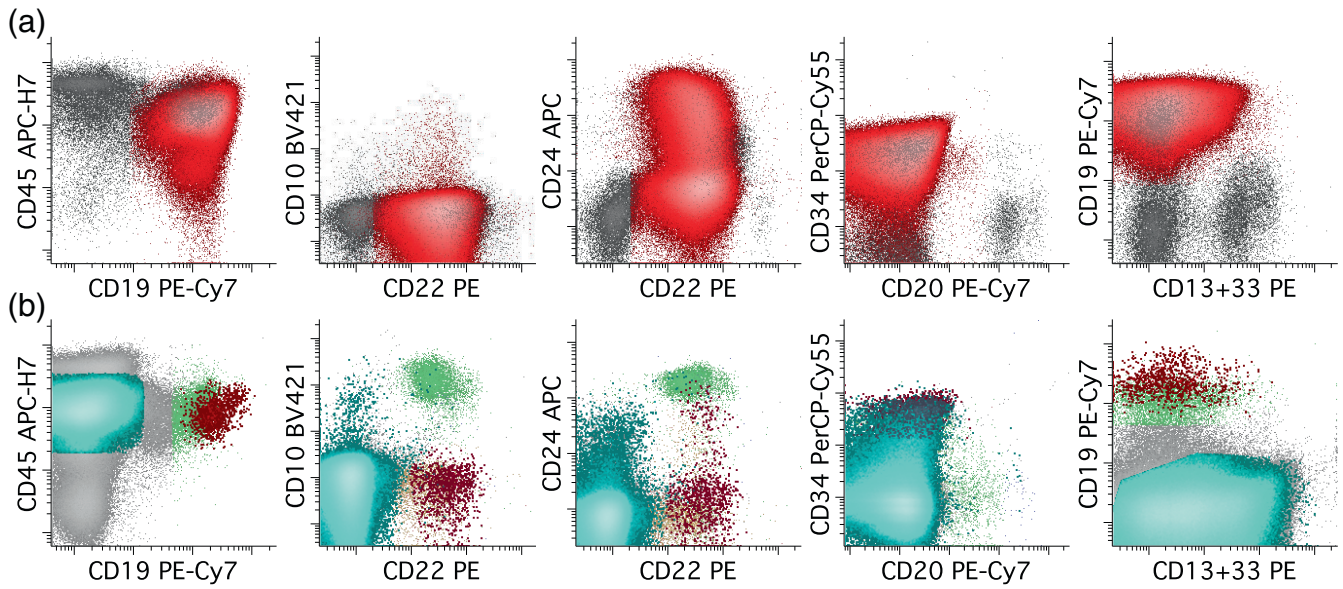

FIGURE 8 Lineage switch post therapy. (a) B-lymphoblastic leukemia, KMT2A rearranged, pretherapy. Abnormal blasts are highlighted in red and demonstrate expression of CD19 and CD22 with variable CD24 and CD34 without CD10. The blasts are largely absent for CD13/CD33 and are negative for CD64 and CD117 (data not shown). (b) The 4 months from diagnosis, now post therapy with blinatumomab. The patient has a small subset of CD19-positive blasts with an immunophenotype similar to that seen in this patient prior to therapy (highlighted in red). The dominant blast population (highlighted in aqua) lacks expression of CD19, CD22, and CD24. This population expresses variable CD13/CD33, and variable CD117 and CD64 (data not shown). Both time points demonstrate evidence of a KMT2A rearrangement. [Color figure can be viewed at wileyonlinelibrary.com]

with daratumumab binding sites, the persistence of daratumumab on the cell surface interferes with CD38 detection by flow cytometry, resulting in apparent loss of CD38 in all CD38-expressing cells. Down-regulation or loss of CD38 expression is an aberrant immunophenotype that is frequently seen in leukemic blasts. Although CD38 is not likely a target for B-ALL therapy, this antigen may be targeted in patients with B-ALL for other reasons. For instance, it has been reported that daratumumab is a potentially effective therapy in autoimmune hemolytic anemia (AIHA) in pediatric patients post HSCT (Schuetz et al., 2018). Figure 9 illustrates an example of a patient with B-ALL in remission who received daratumumab for AIHA. In this setting, normal, regenerating hematogones appear CD38 negative, mimicking leukemic blasts. This phenomenon of epitope masking by daratumumab can be recognized by displaying all viable cells versus CD38, demonstrating absence of CD38 in all CD38-expressing cells including T/NK cells, myeloid cells, and plasma cells, to avoid misinterpretation as residual disease.

BPX-501 T cells are allogeneic donor T cells administered following HSCT to control infection and prevent relapse of hematologic malignancies. BPX-501 cells are genetically modified with an inducible safety switch that can be activated by binding of remiducid to induce apoptosis in the event of graft versus host disease (GvHD) (Di Stasi et al., 2011). The construct contains a truncated CD19 as a marker to identify donor T cells that have been transduced with safety switch successfully. In a patient who received BPX-501 cell infusion following HSCT for B-ALL, flow cytometric assay for MRD evaluation in a marrow sample collected at day 17 post HSCT revealed an unusual population that expresses CD19, CD38 (dim), CD45, and CD58 without CD10 or CD20 (Figure 10). The population raised some concern for residual leukemic blasts initially, but the immunophenotype was different from that identified in the diagnostic sample. Additional assay demonstrated the population expressed T cell markers CD3 and CD7 (Figure 10b) confirming a T cell lineage and representing the therapeutic BPX-501 T cells. The interference of BPX-501 T cells with MRD detection in B-ALL has been reported (Elshoury et al., 2018), posing significant risk for a false-positive MRD result affecting subsequent therapy if the treatment history is not communicated with pathologists. Other therapeutic products, such as T cells transduced with a suicide gene combining target epitopes from both CD20 and CD34 (Philip et al., 2014), may also interfere with MRD detection in CD20-positive lymphoma and CD34-positive acute leukemia.

These examples highlight that details of treatment history is a key factor in flow cytometric data assessment for MRD to ensure accurate interpretation. If the immunophenotype of the neoplastic or background populations does not match the maturation pattern in the setting of standard therapies, it is critical to consider whether targeted therapies may have been employed.

7 | CONCLUSIONS

Targeted immunotherapy has been increasingly used in the treatment of relapsed/refractory B-ALL with improved outcome. These antigen directed immunotherapies often target antigens used in flow cytometric assays for B cell gating or for determining if a population is normal or aberrant. As targeted therapies may induce immunophenotypic shifts of normal and abnormal populations, it is critical in the current therapeutic environment to be aware of the therapeutic agents in use in your patient population and to understand how such agents may impact the immunophenotype of normal progenitors and leukemic

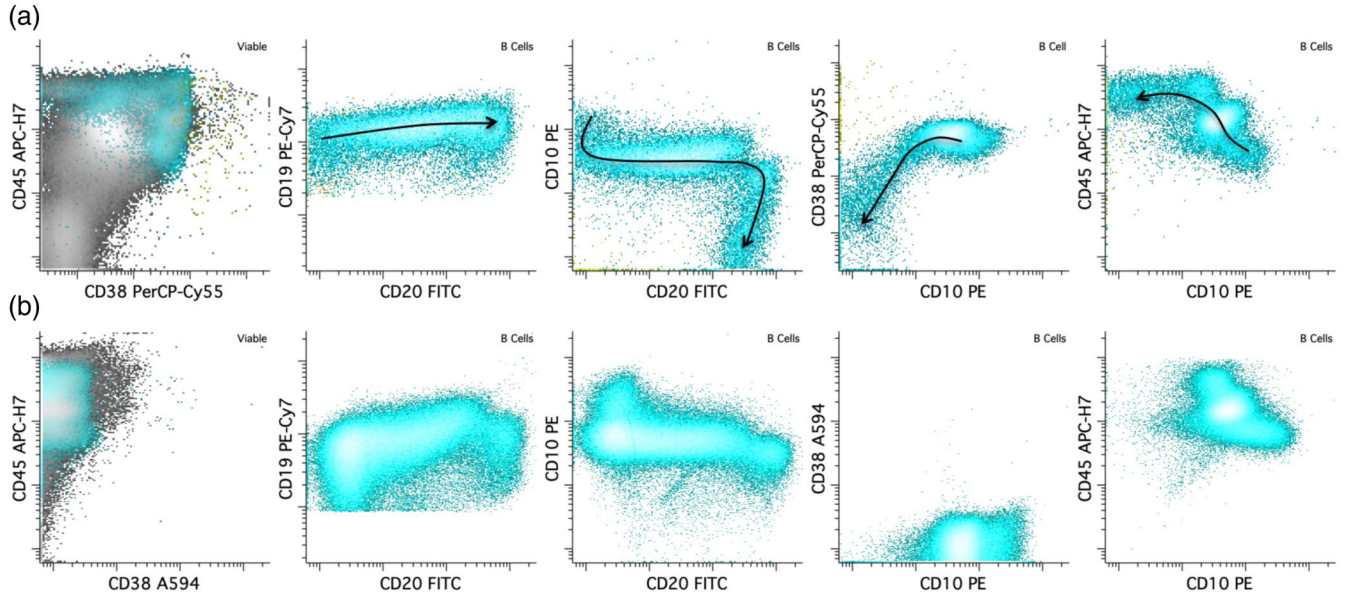

FIGURE 9 Hematogones post anti-CD38 mAb mimicking MRD. (a) Normal B cell maturation. Arrows follow changes in antigen expression with maturation. (b) The hematogones post anti-CD38 mAb lack CD38 expression but with an otherwise normal immunophenotype. [Color figure can be viewed at wileyonlinelibrary.com]

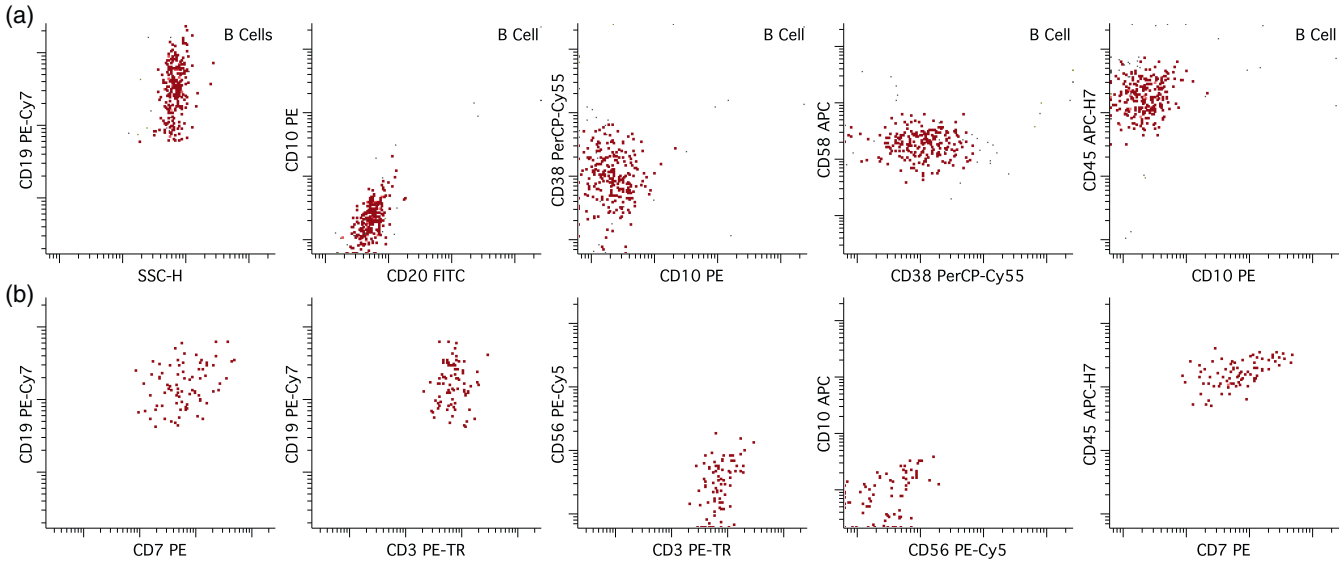

FIGURE 10 Therapeutic cellular products may mimic MRD. (a) BPX-501 cells are genetically modified T cells expressing CD19, mimicking residual B-lymphoblastic leukemia. (b) Additional testing reveals that these cells express T cell markers CD3 and CD7 without CD56, representing BPX-501. [Color figure can be viewed at wileyonlinelibrary.com]

blasts. The modern flow cytometry assay must have significant redundancy in the antibody panel employed, relying on several antigens to recognize B lineage cells, and must utilize several gating strategies for accurate and consistent population identification, even in the setting of antigen escape. If data demonstrates unexpected findings, returning to the clinical picture will allow one to ensure that correct information is acquired, which is critical to achieve the right diagnosis. As we move into the future, it may be useful to identify situations where molecular or alternative genetic methods for MRD assessment may be beneficial to confirm flow cytometric findings. As analysis strategies increase in complexity, there is a growing need for automated computational tools to assist assay standardization in the setting of such complex multiparametric analyses.

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CONFLICT OF INTEREST

All authors declare that they have no conflict of interest.

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